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CAMILA DE SOUSA BEZERRA

Levantamento epidemiológico para as infecções pelo vírus da Anemia Infecciosa Equina e Hepacivírus A em equinos do Nordeste do Brasil

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Camila de Sousa Bezerra

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Tese submetida ao Programa de Pós-Graduação em Ciência e Saúde Animal, da Universidade Federal de Campina Grande, como requisito parcial para obtenção do grau de Doutor em Ciência e Saúde Animal.

Prof. Dr. Sérgio Santos de Azevedo

Dr. Maria Luana Cristiny Rodrigues Silva

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CAMILA DE SOUSA BEZERRA

LEVANTAMENTO EPIDEMIOLÓGICO PARA INFECÇÕES VIRAIS EM EQUINOS DO NORDESTE DO BRASIL

Tese apresentada ao Programa de Pós-Graduação em Ciência e Saúde Animal como pré-requisito para obtenção do título de Doutor em Ciência e Saúde Animal.

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Prof. Dr. Clebert José Alves (Examinador Interno - PPGCSA/UFCG)

Proa. Dra. Carolina de Sousa Américo Batista Santos (Examinadora Interna - PPGCSA/UFCG)

Prof. Dr. Matheus Nunes Weber (Examinador Externo - Universidade FEEVALE)

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RESUMO

O rebanho efetivo brasileiro conta com aproximadamente 5.9 milhões de equinos, dos quais mais de 1.3 milhões pertencem à região Nordeste, onde a atividade possui valor econômico, esportivo e cultural. Na criação de animais, a identificação das infecções circulantes no rebanho, bem como dos aspectos epidemiológicos a elas relacionados, devem ser levados em consideração, visando a elaboração de medidas profiláticas específicas para cada região. Por sua vez, as infecções de etiologia viral, por possuírem alta taxa de transmissão, diferenciação genética e mecanismos de escape do sistema imune do hospedeiro, são responsáveis por percas significativas na equideocultura. Tendo em vista o impacto da ocorrência de infecções virais para a criação de equinos, e para a saúde pública, foram elaborados estudos epidemiológicos para duas infecções virais e uma revisão sistemática de literatura com meta- análise dos dados. O primeiro trabalho objetivou determinar a prevalência e as áreas de maior densidade de animais positivos para o vírus da anemia infecciosa equina (*EIAV*), em quatro estados na região Nordeste do Brasil. O segundo estudo consistiu na determinação da prevalência do hepacivírus equino (*EqHV*) e análise filogenética, nos estados da Paraíba, Pernambuco, Ceará e Rio Grande do Norte, região Nordeste do Brasil. Por fim, foi realizada uma revisão sistemática de literatura e meta-análise dos trabalhos de prevalência do *EqHV*, fornecendo assim uma atualização global da infecção. Para determinar a prevalência de *EIAV*e *EqHV*, foram utilizadas amostras de soro sanguíneo de equinos, cedidas pelo Laboratório Veterinária Diagnósticos – LTDA. A partir dos resultados do capítulo I, foi observado que o *EIAV* é circulante nos quatro estados da região Nordeste do Brasil, sendo o estado de CE o de maior prevalência de animais positivos, assim como áreas com maiores densidades de animais positivos. As regiões de fronteira entre os estados foram as áreas com os maiores aglomeradosde casos positivos. Em relação ao capítulo II, foi possível evidenciar a circulação de *EqHV* na região Nordeste do Brasil. A análise filogenética revelou que a região NS3 de *EqHV* de amostras do Nordeste do Brasil estava intimamente relacionada com cepas isoladas em outras regiões do Brasil, sugerindo a presença de um ancestral comum entre as cepas virais no país. Finalmente, no capítulo III foi observada uma variabilidade das prevalências obtida por continente, além de fatores de risco à infecção pelo *EqHV* associadas indiretamente relacionadas ao manejo dos animais.

Palavras-chave: Anemia infecciosa equina; densidade de casos; hepacivírus equino; sequenciamento genético; revisão sistemática; meta-análise

ABSTRACT

The effective Brazilian herd has approximately 5.9 million horses, of which more than 1.3 million belong to the Northeast region, where the activity has economic, sporting and cultural value. In animal husbandry, the identification of infections circulating in the herd, as well as the epidemiological aspects related to them, must be taken into account, aiming at the elaboration of specific prophylactic measures for each region. In turn, infections of viral etiology, due to their high transmission rate, genetic differentiation and mechanisms of escape from the host's immune system, are responsible for significant losses in equine culture. In view of the impact of the occurrence of viral infections on equine breeding and public health, epidemiological studies were carried out for two viral infections and a systematic literature review with meta-analysis of the data. The first study aimed to determine the prevalence and areas with the highest density of animals positive for the equine infectious anemia virus (*EIAV*) in four states in the Northeast region of Brazil. The second study consisted of determining the prevalence of equine hepacivirus (*EqHV*) and phylogenetic analysis in the states of Paraíba, Pernambuco, Ceará and Rio Grande do Norte, in the Northeast region of Brazil. Finally, a systematic literature review and meta-analysis of *EqHV* prevalence studies were performed, thus providing a global update on the infection. To determine the prevalence of *EIAV* and *EqHV*, blood serum samples from horses, provided by Laboratório Veterinária Diagnósticos – LTDA, were used. From the results of chapter I, it was observed that *EIAV* is circulating in the four states of the Northeast region of Brazil, with the state of CE having the highest prevalence of positive animals, as well as areas with higher densities of positive animals. Border regions between states were the areas with the largest clusters of positive cases. In relation to chapter II, it was possible to evidence the circulation of *EqHV* in the Northeast region of Brazil. Phylogenetic analysis revealed that the NS3 region of *EqHV* from samples from Northeast Brazil was closely related to strains isolated in other regions of Brazil, suggesting the presence of a common ancestor among viral strains in the country. Finally, in Chapter III, a variability of prevalence obtained by continent was observed, in addition to risk factors for *EqHV* infection indirectly associated with the handling of animals.

Keywords: Equine infectious anemia; case density; equine hepacivirus; genetic sequencing; systematic review; meta-analysis

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Lista de Abreviaturas e Siglas

AGID	Agar gel immunodiffusion assay
AP	Apparent prevalence
AST	Aspartate transaminase
cDNA	Complementary DNA
CE	Ceará
CHV	*Canine hepacivirus*
CI	Confidence interval
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
DENV	Dengue virus
DNA	Deoxyribonucleic acid
EIA	Equine infectious anemia
EIAV	Equine infectious anemia vírus
ELISA	Enzyme Linked Immuno Sorbent Assay
EqHV	Equine hepacivirus
ESP	Specificity
GGT	Gamma-glutamyl transferase
GLDH	Glutamate dehydrogenase
GOT	Glutamic-oxaloacetic transaminase
GPT	Glutamate pyruvate transaminase
HCV	*Hepatitis C vírus*
K2P	Kimura two parameters
MAPA	Ministério da Agricultura, Pecuária e Abastecimento
NJ	Neighbor-Joining
OIE	Organização Mundial de Saúde Animal
OR	Odds ratio
PB	Paraíba
PCR	Polymerase Chain Reaction
PE	Pernambuco
PNSE	Programa Nacional de Sanidade de Equídeos
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
RN	Rio Grande do Norte
RNA	Ribonucleic acid
RP	Real prevalence

RT	Reverse transcriptase
SDA	Secretaria de Defesa Agropecuária
SDH	Succinate dehydrogenase
SEN	Sensibility
UFCG	Universidade Federal de Campina Grande
UFPB	Universidade Federal da Paraíba
UFRGS	Universidade Federal do Rio Grande do Sul
USP	Universidade Federal de São Paulo
UTM	Universal Transverse Mercator coordinates
UTR’s	Untranslated regions
YFV	Yellow fever virus
ZIKV	Zika Virus

Lista de Símbolos

%	Porcentagem
R$	Real
>	Maior
<	Menor
≥	Maior ou igual
≤	Menor ou igual
+	Adição
-	Subtração
=	Igualdade
Km	Kilometre
bp	Base pair
n	Número

SUMÁRIO

INTRODUÇÃO GERAL

O rebanho efetivo brasileiro conta com aproximadamente 5.9 milhões de equinos, dos quais mais de 1.3 milhões pertencem à região Nordeste (IBGE, 2019), onde a atividade possui valor econômico, esportivo e cultural.

Na criação de animais, a identificação das infecções circulantes no rebanho, bem como dos aspectos epidemiológicos a elas relacionados, devem ser levados em consideração, visando a elaboração de medidas profiláticas específicas para cada região (ROBERTSON, 2020). Por sua vez, as infecções de etiologia viral, por possuírem alta taxa de transmissão, diferenciação genética e mecanismos de escape do sistema imune do hospedeiro, são responsáveis por percas significativas na equideocultura (PAILLOT, 2020).

Entre as infecções virais de maior impacto na criação de equinos está a anemiainfecciosa equina (EIA), causada por um lentivírus da família *Retroviridae*, subfamília *Orthoretrovirinael* (ICTV, 2019). A infecção pelo vírus da anemia infecciosa equina (*EIAV*) pertence a uma das onze doenças notificáveis específicas para equinos listadas pela Organização Mundial de Saúde Animal (OIE), e a sua ocorrência gera perdas econômicas significativas, uma vez que no Brasil, os animais positivos devem ser sacrificados como medida de controle da infecção (MAPA, 2004).

O *Hepacivirus equino* (*EqHV*) pertence à família *Flaviviridae*, gênero *Hepacivirus* (SMITH et al., 2016) e possui alta identidade genômica com o vírus da hepatite C (*HCV*) (BURBELO et al., 2012), uma das principais causas de hepatite crônica em seres humanos (CHEVALIEZ & PAWLOTSKY, 2006; SHCEEL et al., 2016).

A prevalência de *EqHV* já foi determinada em algumas regiões do Brasil, nas quais foram observados valores que variam de 9,4 a 13,4% (GEMAQUE et al., 2014; FIGUEIREDO et al., 2015; FIGUEIREDO et al., 2018), porém não há estudos sobre a caracterização filogenética e epidemiológica do *EqHV* na região Nordeste, onde a criação de equinos tem grande importância cultural e econômica, devido à popularidade dos esportes equestres como a vaquejada. Trabalhos de caracterização e de sequenciamento genético do *EqHV* colaboram no entendimento da fisiopatologia do *HCV*, o qual é limitado pelos sistemas de cultura celular e pela falta de um modelo animal para estudo direto da infecção (BILLERBECK et al., 2013).

O primeiro relato do *EqHV* ocorreu em 2012 nos EUA, sendo inicialmente identificado como hepacivirus canino (*CHV*). Atualmente estão disponíveis trabalhos realizados no Brasil (GEMAQUE et al., 2014; FIGUEIREDO et al., 2015), África do Sul

(BADENHORST et al., 2018), Japão (MATSUU et al., 2019), China (LU et al., 2016), Coreia do Sul (KIM et al., 2017), Itália (ELIA et al., 2017), França (PRONOST et al., 2017), Alemanha (POSTEL et al., 2016; DEXLER at al., 2013), Reino Unido (LYONS et al., 2012),e Hungria (REUTER et al., 2014). Uma revisão sistemática de literatura sobre o *EqHV* e meta-análise das prevalências identificadas, será útil para avaliar e resumir os dados sobre a infecção, fornecendo assim uma atualização global da circulação do *EqHV*.

A utilização de bancos de amostras em trabalhos de caracterização epidemiológica como fonte de dados, torna viável a identificação das infecções circulantes e incidentes no rebanho, bem como da sua periodicidade, devido a possibilidade de se trabalhar com ummaior número de animais (THRUSFIELD & CHRISTLEY, 2018).

Tendo em vista o impacto da ocorrência de infecções virais para a criação de equinos, e para a saúde pública, foram elaborados estudos epidemiológicos para duas infecções virais e uma revisão sistemática de literatura com meta-análise dos dados. O primeiro trabalho objetivou determinar a prevalência e as áreas de maior densidade de animais positivos para o *EIAV*, em quatro estados na região Nordeste do Brasil. O segundo estudo consistiu na determinação da prevalência e no sequenciamento genético de amostras positivas para o *EqHV*, nos estados da Paraíba, Pernambuco, Ceará e Rio Grande do Norte, região Nordeste doBrasil. Por fim, foi realizada uma revisão sistemática de literatura e meta-análise dos trabalhos de prevalência global do *EqHV*.

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CAPÍTULO I:

Título do capítulo I

True prevalence and spatial distribution of equine infectious anemia virus (EIAV) in horses from Northeast region of Brazil

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True prevalence and spatial distribution of equine infectious anemia virus (EIAV) in horses from Northeast region of Brazil

Camila de Sousa Bezerra[1], Denize Monteiro dos Anjos[2], Brunna Muniz Rodrigues Falcão[1], Cícero Wanderlô Casimiro Bezerra[1], Davidianne de Andrade Moraes[1], Denise Batista Nogueira[3], Maria Luana Cristiny Rodrigues Silva[1] & Sérgio Santos de Azevedo[1]

[1]Federal University of Campina Grande (UFCG), Patos, Paraíba, Brazil. [2]Federal University of Paraíba (UFPB), João Pessoa, Paraíba, Brazil. [3]University of São Paulo (USP), São Paulo, São Paulo, Brazil. CORRESPONDENCE: S.S. de Azevedo [sergio.santos@professor.ufcg.edu.br]. Department of Preventive Veterinary Medicine - UFCG. Avenida Universitária s/n, Santa Cecilia, CEP 58708-110, Patos, PB, Brazil.

ABSTRACT

Backgound: Equine infectious anemia (EIA) is a viral infection, caused by a lentivirus of the Retroviridae family, Orthoretrovirinael subfamily and its occurrence generates significant economic losses due to culling of positive animals as a measure of infection control. The objective of this work was to determine the prevalence of horses positive for EIAV and to identify the occurrence of areas with higher densities of cases in the states of Paraíba (PB), Pernambuco (PE), Rio Grande do Norte (RN) and Ceará (CE), Northeast region of Brazil, during the rainy (May and June) and dry (October and November) periods of 2017 and 2018. ***Materials, Methods & Results:*** Blood serum samples of horses from the states of PB, PE, RN and CE, provided by the Laboratório Veterinária Diagnostics – LTDA, were used. Serological diagnosis of EIA was performed using indirect enzyme-linked immunosorbent assay (ELISA) as a screening test and agar gel immunodiffusion test (AGID) as a confirmatory test. The apparent prevalence was obtained by dividing the number of seroreactive animals by the total

number of animals, while the real prevalence was estimated by adjusting the apparent prevalence, considering the sensitivity (100%) and specificity (98.6%) of the diagnostic protocol used. For the construction of Kernel estimates, the Quartic function was used. In the dry season, of the 1,564 animals sampled, 28 were serologically positive, of which 19 belonged to the state of Ceará, seven to Paraíba and two to Rio Grande do Norte. In 2018, it was observed that, during the rainy season, 26 of the 1,635 horses were seroreactive, with 19 cases resulting from Ceará, four from Paraíba and three from Pernambuco. In the dry season, 32 of the 1,526 animals were seroreactive to EIAV, of which 26 were from Ceará, three from Paraíba, one from Rio Grande do Norte and two from Pernambuco. In the dry period of 2017, the CE had a real prevalence of 1.22% (95% CI = 0.05 - 2.99%). In 2018, during the rainy season, prevalences of 0.03% (95% CI = 0 - 1.18%) were identified in CE and 1.69% (95%CI = 0 - 8.38%) in PE. Regarding the 2018 dry period, a prevalence of 1.32% (95% CI = 0.26
- 2.84%) was found in the state of CE. In both dry and rainy periods of 2017, the presence of spatial clusters of animals positive for EIA was observed, mainly in the border areas among the states of CE, PE, PB and RN. In 2018, there was a variation in the distribution of areas with higher densities of cases between the rainy and dry periods.

Discussion: The state of CE had the highest prevalence of positive animals and the presence of areas with higher densities of EIA cases in both climatic periods, in the years 2017 and 2018. In some municipalities of the CE, important sporting events of agglomeration ofanimals take place, which can favor the transmission of EIAV by facilitating the contact of infected and susceptible animals. Population density may be a factor associated with the higher prevalence observed in this region, as it has the second largest herd among the states studied. Higher densities indirectly contribute to the occurrence of infectious diseases, as they favor the contact of infected and susceptible animals. The occurrence of higher densities of cases in the border areas of the states of PE, RN, CE, and PB may be related to the greater

movement of animals in these regions, favoring the indirect contact of infected horses with susceptible ones. The observed results demonstrate the circulation of the EIAV in four states in the Northeast region of Brazil.

Keywords: Equine infectious anemia virus, prevalence, spatial clusters

INTRODUCTION

Equine infectious anemia (EIA) is a viral infection caused by a lentivirus of the Retroviridae family and Orthoretrovirinael subfamily [10]. Similar to other retroviruses, the EIA virus (EIAV) performs reverse transcription by the reverse transcriptase enzyme (RT), producing proviral DNA, which is integrated into the host cell DNA [7,11], allowing the occurrence of persistently infected animals.

The prevalence of EIAV has already been described in some Brazilian states through seroprevalence studies, in which variable viral circulation data were obtained, from 0.44% in Minas Gerais [1] to 17.2% in the state of Mato Grosso [2].

EIAV infection belongs to one of the eleven notifiable diseases specific to horses listed by the World Organization for Animal Health (OIE), and its occurrence generates significant economic losses, since according to Normative Instruction N^{o}. 45 of the Ministry of Agriculture, Livestock and Supplies (MAPA) in Brazil, positive animals should be culled as a measure of infection control [12].

The effective Brazilian herd has approximately 5.9 million horses, of which more than 1.3 million belong to the Northeast region [9], where the activity has economic, sporting and cultural values, however there is no comprehensive work on the circulation of the EIAV in this region. Thus, the objective of this study was to determine the prevalence of animals positive for EIAV and to identify the occurrence of areas with higher densities of cases of

infection in the states of Paraíba (PB), Pernambuco (PE), Rio Grande do Norte (RN) and Ceará (CE) in Northeast region of Brazil, during the rainy (May and June) and dry (October and November) periods of 2017 and 2018.

MATERIAL AND METHODS

Study area and sampling

The samples of this study were collected in the states of Paraíba (7°14'23.86"S; 36°46'55.02"W), Pernambuco (8°48'49.39"S; 36°57'14.79"W), Rio Grande do Norte (5°24'9.29"S; 36°57'14.79"W) and Ceará (5°29'54.06"S; 39°19'9.95"W), located in the Northeast region of Brazil (Figures 1 and 2), characterized by having a semiarid climate, irregular rainy season and high temperatures [16]. The effective herd of horses in each state is 62,305 in Paraíba, 125,968 in Pernambuco, 67,444 in Rio Grande do Norte and 121,110 in Ceará [9].

All samples were provided by the Laboratório Veterinária Diagnostics – LTDA, located in the municipality of Catolé do Rocha – PB. The laboratory is accredited by MAPA, through SDA Ordinance No. 120, of July 9, 2014, to carry out diagnostic tests for EIA and glanders. In order to assess the prevalence of EIAV-positive animals according to the climaticseason, data were collected from the rainy season (May and June) and the dry season (October and November), from the years 2017 and 2018 [8]. In total, 6,566 samples were used, with 1,841 in the rainy season of 2017 and 1,635 in the dry season of the same year; the number of samples used in the rainy and dry seasons in 2018 was 1,564 and 1,526, respectively (Table1).

Serological diagnosis

The serological diagnosis of EIA was performed at Laboratório VeterináriaDiagnostics – LTDA, using the indirect enzyme-linked immunosorbent assay (ELISA)[1] as a screening test and agar gel immunodiffusion test (AGID)[2] as a confirmatory test, following the recommendations of the manufacturers of commercial kits. Both tests are deemed official for the detection of EIAV antibodies, according to the National Equine Health Program (PNSE) [5].

Data analysis

Apparent and real prevalences were calculated according to Noordhuizen et al. [13]. Apparent prevalence was obtained by dividing the number of seroreactive animals by the total number of animals, while real prevalence was estimated by adjusting the apparent prevalence considering sensitivity (100%) and specificity (98.6%) of the diagnostic protocol used [14] according to this formula:

$$RP = \frac{(AP + ESP - 1)}{(SEN + ESP - 1)}$$

Where,

RP = real prevalence

AP = apparent prevalence

ESP = specificity

SEN = sensitivity

Determination of case densities

The maps were made from a database prepared in Excel spreadsheets, with subsequent spatialization in the QGIS vs 3.10 (LTR) software, forming point shapifile files for the construction of Kernel density maps for the occurrence of positive cases within the

municipalities. All data were georeferenced with Universal Transverse Mercator coordinates – UTM and Datum Sirgas2000.

For the construction of Kernel estimates, the Quartic function was used, which represents events within a region of influence, estimating them by the distance of each one to a location of interest [6]. For the Kernel estimation, a radius of 50km was adopted between the positive cases within each municipality.

RESULTS

Figures 1 and 2 show the spatial distribution of the investigated municipalities. In 2017, 207 municipalities were evaluated in the dry season and 207 in the rainy season, and in 2018, 197 and 181 municipalities, respectively. During the 2017 rainy season, 22 of the 1,841 animals tested were positive for EIAV (12 from Ceará, eight from Paraíba and two from Rio Grande do Norte). In the dry season, of the 1,564 animals sampled, 28 were positive, of which19 belonged to the state of Ceará, seven to Paraíba and two to Rio Grande do Norte. Therewas no positive result in the state of Pernambuco in 2017. In 2018, 26 of the 1,635 horses were seroreactive, being 19 in Ceará, four in Paraíba and three in Pernambuco, with no positive cases in the state of Rio Grande do Norte. In the dry season, 32 of the 1,526 animals were seroreactive, of which 26 were from Ceará, three from Paraíba, one from Rio Grande do Norte and two from Pernambuco (Table 1).

Considering the sensitivity (100%) and specificity (98.6%) of the diagnostic protocol used [14], the real prevalences for each state were determined (Table 1). The highest prevalences were 1.22% (95% CI = 0.05 - 2.99%) in the 2017 dry period, and 1.32% (95% CI = 0.26 - 2.84%) in the 2018 dry period, both for the CE.

Figures 3 and 4 show the densities of EIA cases in the dry and rainy periods of 2017 and 2018, respectively, in which the lowest density of cases (1) is visualized in blue and the highest (4) in red.

In both the dry and rainy periods of 2017, the presence of agglomerations of animals positive for EIA was observed, mainly in the border areas among the states of CE, PE, PB and RN. The highest densities of cases were located in the intermediate regions of Quixadá (dry period), Iguatu and Juazeiro do Norte, state of CE, and in the intermediate region of Patos, state of PB. There were also agglomerations in the intermediate regions of João Pessoa in PB, Fortaleza in CE and Mossoró (rainy season) in RN.

Regarding the 2018 data, there was variation in the distribution of case densities between the rainy and dry periods. In the rainy season, the EIAV cases belong to the intermediate regions of Petrolina, in the state of PE, to Sousa-Cajazeiras and Patos, in the state of PB, and to the regions of Caicó and Mossoró, in the state of RN. The state of CE has the largest number of clusters of cases in the rainy season, distributed in the intermediate regions of Juazeiro do Norte, Iguatu, Crateús, Fortaleza and Quixadá, the latter being the one with the highest density of cases. In the dry period, two denser areas are evidenced, one in the border areas of the states of PE, PB, RN and CE and the other in the regions of Sobral and Fortaleza in the state of CE, in which there was a higher density of EIA cases.

DISCUSSION

In the present survey, a representative number of animals from the four sampled states was used, however, the absence of more than one laboratory as a data source was a limiting factor in the sampling design, with areas from which no data were obtained. However, the use of a serum bank is important in epidemiological characterization studies [15], as it allows the

identification of circulating infections in the herd, as well as their periodicity, in addition to the evaluation of economic losses related to it.

To calculate the true prevalence, the sensitivity and specificity values of the diagnostic test used are taken into account, as well as the apparent prevalence, so that the proportions of false-positive and false-negative results can be quantified. In most sample groups, the real prevalence was 0, a reflection of possible false-positives. However, the 95% CI allows for a better assessment of the real prevalence, since it determines the variation of the value found, for example, the real prevalence of the 2017 dry period in the state of Paraíba was equal to zero, however the 95% CI was from 0 - 0.39, indicating a possible variation in prevalence between these values.

Among the four states studied, the CE had the highest prevalence of positive animals (Table 1) and the highest densities of EIA cases in both climatic periods, in 2017 and 2018. In some municipalities in the state of Ceará, such as Jaguaribe, Brejo Santo, Juazeiro do Norte, Mauriti, Milagres and Aurora, there are important sporting events of agglomeration of animals coming from several regions of the state and neighboring states. Such events must respect prophylactic measures for infections [4], such as the mandatory negative test for EIA. The carrying out of clandestine agricultural activities is frequent and because there is no inspection of these events, they are carried out without respecting the established sanitary measures, thus favoring the transmission of the EIAV.

Population density may be a factor associated with the higher prevalence observed in the state of CE, since it has the second largest herd among the states used in this study [9]. Higher densities indirectly contribute to the occurrence of infectious diseases, as they favor the contact of infected and susceptible animals, and it has been identified as a risk factor for other diseases [3]. In addition, until the end of 2019, in the state of Ceará no surveillance cares was carry out in areas of EIAV outbreaks, such as blood collection from all equines on

the property, which may have contributed to the maintenance of viral circulation in this region.

The occurrence of higher densities in the border areas of the states of PE, RN, CE, and PB may be related to the greater movement of animals in these regions, favoring the indirect contact of infected horses with susceptible ones. Similar data was observed in a study on the epidemiological characterization of vesicular stomatitis in the state of PB, in which two spatial clusters were observed, both in state boundary areas, the first between the states of CE and PB, and the second between RN and PB [3].

CONCLUSION

It's concluded that EIAV is circulating in horses from four states of Northeast region of Brazil, with the state of CE being the one with the highest prevalence of positive animals, as well as areas with higher densities of positive animals. Border regions among the states were areas with the greatest clusters of positive cases, thus it is necessary to reinforce the control of the movement of animals and preventive measures against infection in these places.

MANUFACTURERS

[1] IDEXX Laboratories Inc. Westbrook, ME, USA.

[2] Laboratórios Bruch Ltda. São Paulo, SP, Brazil.

Declaration of interest. The authors inform that there are no conflicts of interest. Authors are solely responsible for the content of the article.

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FIGURE LEGENDS

Figure 1. Distribution of properties tested for EIA, in the states of PB, PE, CE and RN, in the rainy and dry season of 2017.

Figure 2. Distribution of properties tested for EIA, in the states of PB, PE, CE and RN, in the rainy and dry season of 2018.

Figure 3. Distribution of densities of seroreactive animals for EIA, in the states of PB, PE, CE and RN, in the rainy and dry season of 2017.

Figure 4. Distribution of densities of seroreactive animals for EIA, in the states of PB, PE, CE and RN, in the rainy and dry season of 2018.

Table 1. Seroprevalence of EIA in horses from the states of PB, CE, RN and PE, in the rainy (May and June) and dry (October and November) seasons of 2017 and 2018.

States	2017								2018							
	Rainy season				Dry season				Rainy season				Dry season			
	Tested	Positive	Prevalence %	95% CI	Tested	Positive	Prevalence %	95% CI	Tested	Positive	Prevalence %	95% CI	Tested	Positive	Prevalence %	95% CI
PB	639	8	0	0 - 0.46	601	7	0	0 - 0.39	400	4	0	0 - 0.55	345	3	0	0 - 0.54
CE	782	12	0	0 - 0.68	595	19	1.22	0.05- 2.99	935	19	0.03	0 - 1.18	790	26	1.32	0.26 - 2.84
RN	294	2	0	0 - 0.46	229	2	0	0 - 1.15	218	0	0	0	265	1	0	0 - 0.11
PE	126	0	0	0	139	0	0	0	82	3	1.69	0 - 8.38	126	2	0	0 - 3.68
Total	1,841	22	0	0 - 0.2	1,564	28	0	0 - 0.59	1,635	26	0	0 - 0.33	1,526	32	0.1	0 - 0.96

Figure 1. Distribution of properties tested for EIA, in the states of PB, PE, CE and RN, in the rainy and dry season of 2017.

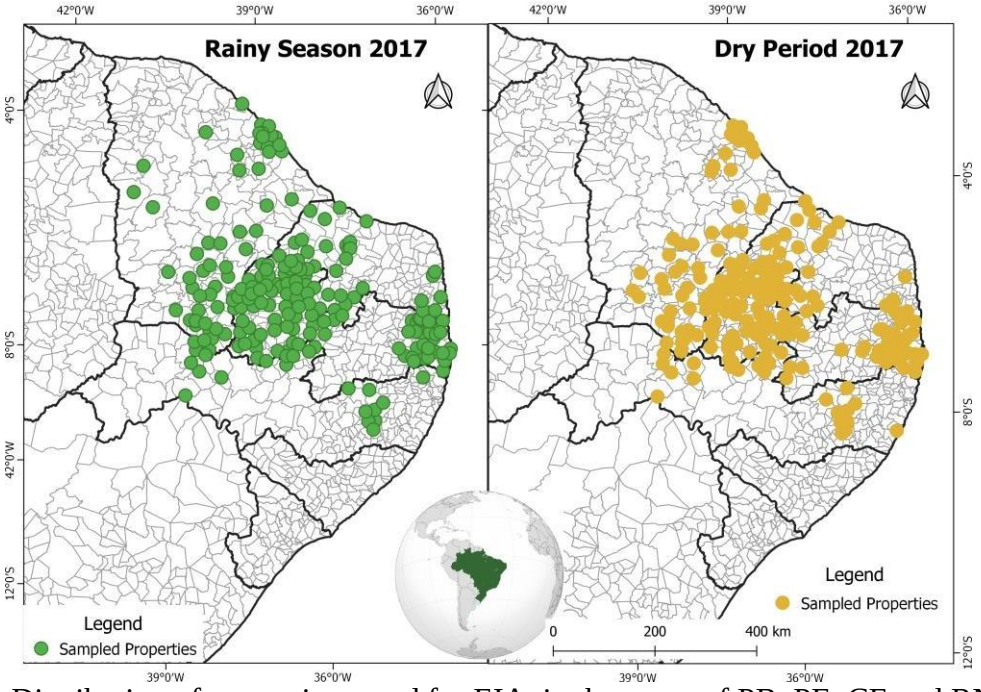


Figure 2. Distribution of properties tested for EIA, in the states of PB, PE, CE and RN, in the rainy and dry season of 2018.

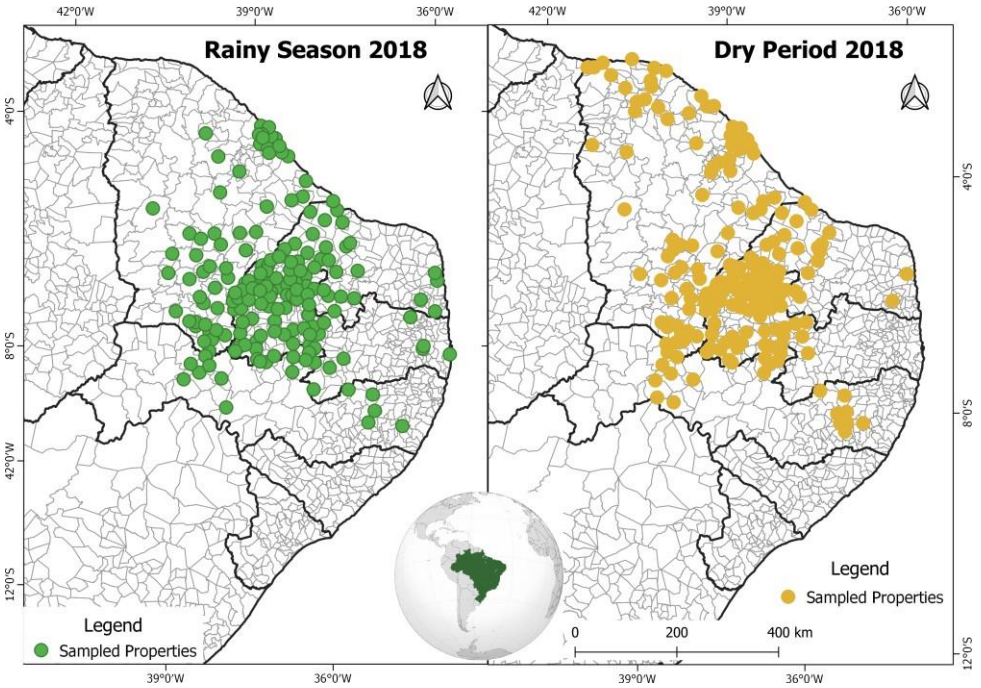


Figure 3. Distribution of densities of seroreactive animals for EIA, in the states of PB, PE, CE and RN, in the rainy and dry season of 2017.

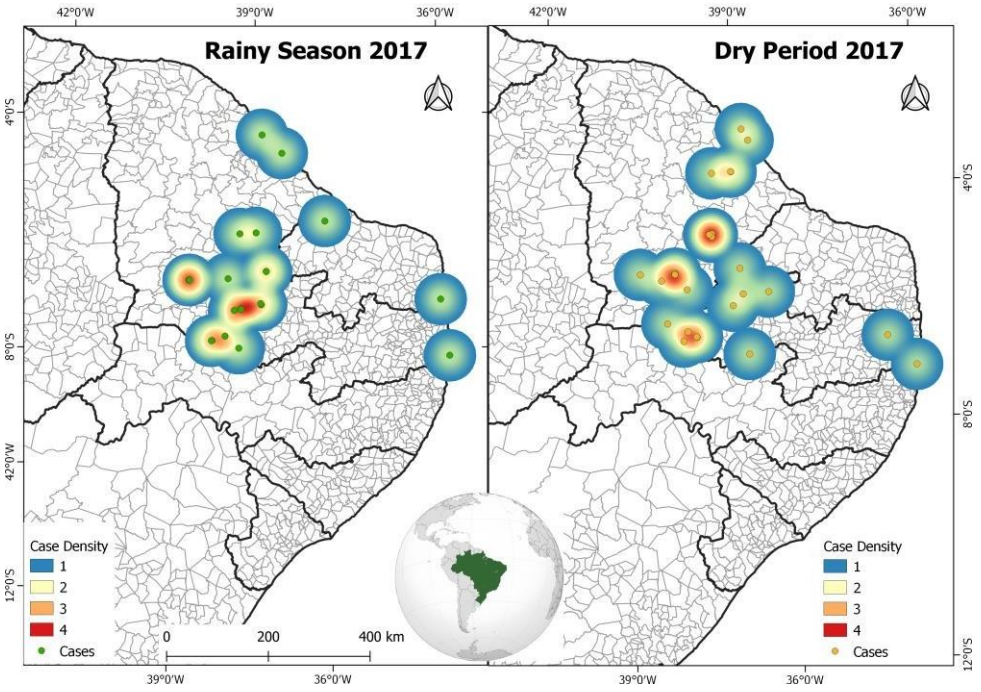
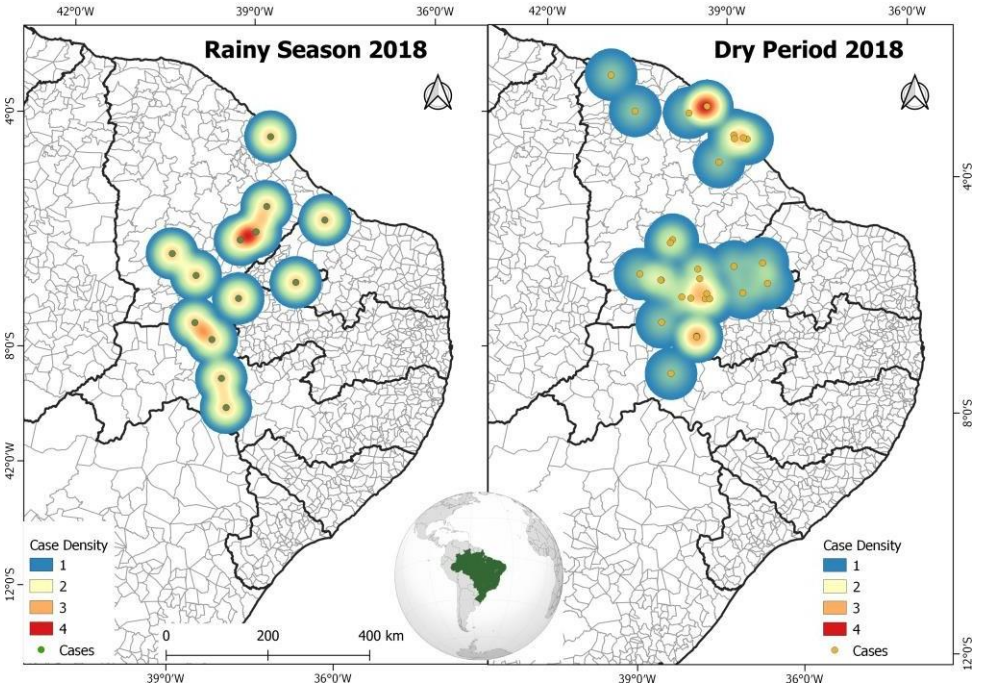


Figure 4. Distribution of densities of seroreactive animals for EIA, in the states of PB, PE, CE and RN, in the rainy and dry season of 2018.



CAPÍTULO II:

Título do capítulo II

Epidemiological investigation of equine hepacivirus in horses from Northeastern Brazil

(Infection, Genetics and Evolution, Qualis: A2, Fator de Impacto: 3.342)

Research papers

Epidemiological investigation of equine hepacivirus in horses from Northeastern Brazil

Camila de Sousa Bezerra[a], Mariana Soares da Silva[b], Matheus Nunes Weber[b], Denize Monteiro dos Anjos[c], Brunna Muniz Rodrigues Falcão[a], Cláudio Wageck Canal[b], Clebert José Alves[a], Carolina de Sousa Américo Batista Santos[a], Maria Luana Cristiny Rodrigues Silva[a], Sérgio Santos de Azevedo[a*].

[a]Federal University of Campina Grande (UFCG), Patos, Paraíba, Brazil.

[b]Federal Universidity of Rio Grande do Sul (UFRGS), Porto Alegre, Rio Grande do Sul, Brazil.

[c]Federal University of Paraíba (UFPB), João Pessoa, Paraíba, Brazil.

*Corresponding author. E-mail address: sergio.santos@professor.ufcg.edu.br (S.S. de Azevedo). Department of Preventive Veterinary Medicine - UFCG. Avenida Universitária s/n, Santa Cecilia, CEP 58708-110, Patos, PB, Brazil.

Abstract

Equine Hepacivirus (*EqHV*) belongs to the *Flaviviridae* family, genus *Hepacivirus*, which is the closest homolog of the *hepatitis C virus* (*HCV*). The prevalence of *EqHV* has already been determined in some regions of Brazil, however there are no studies on the phylogenetic and epidemiological characterization of the *EqHV* in the Northeast region, where equine breeding has great cultural and economic importance, The objective of the present work was to determine the prevalence of *EqHV* in four states in Northeastern Brazil, carry out a phylogenetic analysis of the RNA+ samples and verify the correlation of positivity with the climatic season, sex and age of the animals. 768 equine samples were used, from the states of Paraíba (PB), Pernambuco (PE), Rio Grande do Norte (RN) and Ceará (CE), collected in 2017 and 2018. The sample population consisted of 293 males and 473 females, aged <2 to 20 years. In order to assess the number of positive animals per climatic season, samples from the rainy (May and June) and dry (October and November) periods of the two years were selected. The prevalence of positive *EqHV* horses was 2.2% (17/768), Paraíba being the state with the highest prevalence of reactive animals (4.7%) among the others. Phylogenetic analysis revealed that the NS3 region of *EqHV* from samples from Northeastern Brazil was closely related to strains isolated in other regions of Brazil, suggesting the presence of a common ancestor among viral strains within the country. From the data obtained in the present work, it was possible to evidence the circulation of *EqHV* in the Brazilian Northeast region.

Key-words: Equine hepacivirus, prevalence, phylogenetic analysis.

1. Introduction

Equine Hepacivirus (*EqHV*), also called hepacivirus A (HAV), belongs to the *Flaviviridae* family, genus *Hepacivirus* (ICTV, 2019), which is the closest homolog of the *hepatitis C virus* (*HCV*) (Hartlage et al., 2016), leading cause of chronic hepatitis in humans

(WHO, 2021). This similarity between *EqHV* and *HCV* indicates the possibility of a common ancestor not yet identified (Pybus and Thézé, 2016).

The hepacivirus genome is formed by two untranslated regions (UTR's) the 5'UTR and the 3'UTR, and a single large open reading frame (ORF) that encodes a unique polyprotein which is cleaved into at least 10 products: the core structural protein (C), two envelope proteins (E1 and E2), non-structural assembly proteins (p7 and NS2) and other non-structural proteins involved in replication (NS3, NS4A, NS4B, NS5A and NS5B) (Penin et al., 2004; Moradpour et al., 2007). As they are highly conserved regions, phylogenetic analysis based on nucleotide sequences of the NS5B and NS3 genes has produced similar results to those obtained by complete genome sequencing (Smith et al., 2016).

The first report of *EqHV* occurred in the USA in 2012, from dogs with respiratory diseases, being initially called canine hepacivirus (*CHV*) (Kapoor et al., 2011). However, horses were later shown to be the primary hosts of *EqHV* (Burbelo et al., 2012). Since then, the circulation of *EqHV* has been described in different countries, such as the United States (Ramsay et al., 2019), South Africa (Badenhorst et al., 2018), Morocco (Abbadi et al., 2021), Japan (Matsuu et al., 2019), China (Lu et al., 2017), South Korea (Kim et al., 2017), Mongolia (Date et al., 2020), Italy (Elia et al., 2017), France (Pronost et al., 2016), Germany (Dexler at al., 2013; Postel et al., 2016), United Kingdom (Lyons et al., 2012) and Hungary (Reuter et al., 2014).

The prevalence of *EqHV* has already been determined in some regions of Brazil, in which values ranging from 9.4 to 13.4% were observed (Gemaque et al., 2014; Figueiredo et al., 2015; Figueiredo et al., 2018), however there are no studies on the phylogenetic and epidemiological characterization of the *EqHV* in the Northeast region, where equine breeding has great cultural and economic importance, due to the popularity of equestrian sports such as vaquejada. Thus, the objective of the present work was to determine the prevalence of *EqHV* in four states in Northeastern Brazil, carry out a phylogenetic analysis of the RNA+ samples and verify the correlation of positivity with the climatic season, sex and age of the animals.

2. Material and methods

2.1 Animals and sampling

The equine sera used in this study were provided by the Laboratório Veterinária Diagnostics – LTDA, located in the municipality of Catolé do Rocha, state of Paraíba, Northeast region of Brazil. The samples were collected in 2017 and 2018, in the states of

Paraíba (PB), Pernambuco (PE), Rio Grande do Norte (RN) and Ceará (CE). The number of selected samples was estimated using the formula for simple random samples, considering a confidence level of 95%, an expected prevalence of 50% and an error of 5%. (Thrusfield and Christley, 2018). A total of 384 animals per year were used, totaling 768 in both years. In order to evaluate the number of positive animals per climatic season, samples from the rainy (May and June) and dry (October and November) periods were selected. The distribution of the sample population is shown in Figures 1 and 2.

2.2 RT-PCR and Sanger sequencing

Sera were aliquoted into pools of 12 samples each, resulting in a total of 64 pools. Pools were subjected to reverse transcriptase reaction, followed by polymerase chain reaction (RT-PCR) specific for *EqHV*. cDNAs were synthesized using GoScript™ Reverse Transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions. *EqHV*-specific PCR was performed using NS3OF (ATHTGTGATGARTGCCAYAGYAC) / NS3OR (TAGTAGGTBACAGCRTTAGCYCC) primers which amplified approximately 250 bp of NS3 (Lu et al., 2016).

PCR amplicons were purified using the Quick PureLink™ PCR Purification Kit (Invitrogen, Carlsbad, CA, USA). Sequenced with an ABI PRISM 3100 Genetic Analyzer using a BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

2.3 Phylogenetic and data analysis

Sequence consensuses were assembled using Geneious software. Representative nucleotide sequences of known hepaciviruses were obtained from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) and aligned with the sequences identified in the present study using the MUSCLE software (Edgar, 2004). The phylogenetic trees were generated by the Neighbor-Joining (NJ) method and the confidence levels of the branches nodes were obtained by the analysis of 1,000 bootstrap repetitions. The statistical model selected was the Kimura-2 Parameters (K2P). Phylogenetic trees were reconstructed using SeaView software (Gouy et al., 2010).

3. Results

3.1 EqHV RNA

Of the 64 pools tested for *EqHV*, 11 were RNA+. Subsequently, each pool was individually opened, in which 2.2% (17/768) of RNA+ animals were identified for *EqHV*, of which 11 corresponded to the year 2017 and six to the year 2018. There was no statistical difference in prevalence between seasons in any state per year ($P > 0.05$). Overall state of Paraíba had the highest prevalence of positive animals (4.7%), which was statistically different from Rio Grande do Norte and Pernambuco. There was no statistical difference among CE, RN and PE (Table 1).

3.2 Age and gender association analysis

The age and gender of the sample population and positive animals are shown in Table 2. In total, 293 males and 473 females were sampled, with ages ranging from < 2 to 20 years. Considering the number of positives by the total number of animals tested in each age group, a higher prevalence of positivity was observed in horses aged less than or equal to 2 years; likewise, males (2.7%) had higher positivity than females (1.9%). However, there was no statistical difference ($P >0.05$).

3.3. Phylogenetic analysis

Of the 17 positive samples, 11 were viable for genetic sequencing (Table 3). The data from the phylogenetic analysis are shown in Figure 3. The sequences identified in this work were organized into two branches. In the first branch, ten sequences were grouped, which showed a strong relationship with two isolates from this work (155_Eq_Patos_PB_BR_2017 and 134_Eq_Santa_Rita_PB_2017) with those described in the state of Pará, northern region of Brazil (KJ469447, KJ469450). There was relative proximity to isolates from Italy, China, New Zealand and the United Kingdom. A single sequence was clustered at the second branch, being strongly related to an isolate from the North region of Brazil (KJ469449) and considerably close to sequences described in Egypt. The grouping of sequences among themselves was also observed.

BLASTn analysis of the NS3 region showed that the contig shared 97.24% nucleotide sequence identity with a Brazilian isolate (7_Eq_Cajazeiras_PB_BR_2018 and KJ469466) and 95.88% with a sequence identified in Egypt (51_Eq_Mossoro_RN_BR_2017 and MG407614).

4. Discussion

The prevalence of *EqHV* positive animals in the sampled population was 2.2%, below to the values described in countries such as Mongolia (47.2%) (Date et al., 2020), Korea (18.9%) (Kim et al., 2017), Japan (17.7%) (Matsuu et al., 2015), Morocco (10.5%) (Abbadi et al., 2021), South Africa (7.9%) (Badenhorst et al., 2018), France (5.6%-6.2%) (Pronost et al., 2016a, 2016b), Italy (4.7%) (Elia et al., 2017), Austria (4.2%) (Badenhorst et al., 2019) and China (3.4%-9.0%-31.7%) (Lu et al., 20216; Wu et al., 2020; Chen et al., 2021). In other countries, however, prevalence rates were close to or lower than those obtained in this study, such as in the United Kingdom (0.9%-2.1%) (Lyons et al., 2012; Lyons et al., 2014), Germany (2.4% -2.5%) (Pfaender et al., 2015; Schlottau et al., 2018) and USA (2.9%) (Ramsay et al., 2019).

Circulation of *EqHV* has been described in other Brazilian states and values above 2.2% were observed in all of them. In the North (Pará) 9.4% of the animals were reactive to *EqHV*. (Gemaque et al., 2014). In the Southeast (Rio de Janeiro and Espírito Santo) and Midwest (Mato Grosso do Sul) 13.4% of the animals were positive (Figueiredo et al., 2015). Finally, a study conducted in the Southeast region (Rio de Janeiro) found a prevalence of 13.4% for *EqHV* (Figueiredo et al., 2018). Based on these values, we can state that the prevalence of viremic animals in the Northeast region of Brazil is relatively lower than those identified elsewhere.

Among the states sampled, Paraíba had the highest prevalence of positive animals (4.7%), which was statistically equal to the prevalence identified in Ceará (2.6%). The forms of transmission of *EqHV* have not yet been fully elucidated, however, iatrogenic and vertical transmission has been observed in some studies (Gather et al., 2016; Tomlinson et al., 2019). For this reason, the presence of the virus in the equine herd may be closely related to the inefficiency of sanitary care during the handling of animals, such as sharing gloves, needles or other material with contaminated biological products. Since the variables related to animal management are not available in this study, it is not possible to infer which factors may be related to a higher proportion of viremic animals in PB and CE. However, the holding of animal agglomeration events in the border areas of the states is frequent and when carried out clandestinely, disobeying the necessary prophylactic requirements, they can facilitate the circulation of infectious agents such as *EqHV*. Thus, it is suggested that the similarity between the prevalence found in the states of PB and CE is a reflection of the movement of animals between these two regions.

Some virus in the *Flaviviridae* family can be transmitted by hematophagous vectors, such as dengue virus (DENV), yellow fever virus (YFV), Zika virus (ZIKV) among others

(Huang et al., 2014). For this reason, the highest incidences of these infections occur during the hottest times of the year, when there are optimal conditions for the proliferation of vectors. With the objective of verifying the levels of viral circulation between the climatic seasons, samples from the rainy and dry periods were selected, however no statistical difference was observed between the obtained prevalences. These results agree with the experiment by Badenhorst et al. (2019), in which of the 5,000 mosquitoes tested, none were positive for *EqHV*, with no evidence of vector transmission.

Considering the variable age of the animals, despite no statistical significance, the highest prevalence of positive RNA+ was 5.1% in horses ≤ 2 years. The susceptibility of young animals to *EqHV* infection is related to the frequent handling of young animals. Such information has already been supported by some authors. Figueiredo et al. (2019) identified young mares with a higher risk of *EqHV* infection, (OR = 7.4; p = 0.0195), possibly due to reproductive management practices in these animals. Similarly Matsu et al. (2015) observed that the number of positive animals between 1 and 2 years of age was significantly higher than the other groups, which according to the authors was related to the transport of these animals to participate in sporting events, thus having a greater chance of exposure.

Unlike what has been shown in other studies, in which females were at higher risk for *EqHV* infection (Reichert et al., 2017; Figueiredo et al., 2018; Abbadi et al., 2021), in this work the highest prevalence by sex were from males (2.7%).

The phylogenetic analysis revealed that the NS3 region of the *EqHV* from samples from the Northeast region of Brazil were grouped into two branches, closely related to the strains of the Northern region of Brazil (Gemaque et al., 2014) and generically to sequences described in Italy, China, Nova Zealand and the United Kingdom. The proximity of the sequences to each other was also observed. These data suggest the occurrence of a common ancestor among viral strains in Brazil.

5. Conclusion

From the data obtained in the present work, it was possible to evidence the circulation of *EqHV* in the Brazilian Northeast region. Phylogenetic analysis revealed that the NS3 region of *EqHV* from samples from Northeastern Brazil was closely related to strains isolated in other regions of Brazil, suggesting the presence of a common ancestor among viral strains within the country. Epidemiological investigations with variables aimed at sanitary

management practices, as well as information on the transit of animals, could clarify the different prevalences found in Brazilian regions and in other countries.

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FIGURE CAPTIONS

Fig. 1. Distribution of the sample population during the 2017 dry and rainy periods.

Fig. 2. Distribution of the sample population during the 2018 dry and rainy periods.

Fig. 3. Phylogenetic reconstruction based on the partial nucleotide sequences of the NS3 gene of equine hepacivirus (EqHV). The tree was constructed under Neighbor-Joining inference, using the K2P model, in 1,000 bootstrap replicates. Sequences reported in the present study are highlighted with ▲.

Table 1. Prevalence of RT-PCR positive animals for EqHV in the states of PB, CE, RN and PE, in the rainy (May and June) and dry (October and November) periods of 2017 and 2018.

States	2017						2018						Total		
	Rainy season			Dry season			Rainy season			Dry season					
	Tested	Positive	Prevalence %	Tested	Positive	Prevalence %	Tested	Positive	Prevalence %	Tested	Positive	Prevalence %	Tested	Positive	Prevalence (%)
PB	48	3	6.3^{A}	48	1	2.1^{A}	48	3	6.3^{A}	48	2	4.2^{A}	192	9	4.7^{A}
CE	48	1	2.1^{A}	48	4	8.3^{A}	48	0	0^{A}	48	0	0^{A}	192	5	2.6^{A,B}
RN	48	1	2.1^{A}	48	1	2.1^{A}	48	0	0^{A}	48	0	0^{A}	192	2	1.0^{B}
PE	48	0	0^{A}	48	0	0^{A}	48	1	2.1^{A}	48	0	0^{A}	192	1	0.5^{B}
Total	192	5	2.6	192	6	3.1	192	4	8.3	192	2	1.3	768	17	2.2

Table 2. Summary of animals population study.

Variable	Categories	No. of tested animals	No. of positive animals (%)
Age (years)	≤ 2	98	5 (5.1)
	> 2 ≤ 10	590	10 (1.7)
	> 10 ≤ 20	79	2 (2.5)
	> 20	1	0 (0)
Gender	Male	293	8 (2.7)
	Female	473	9 (1.9)
	Not identified	2	0 (0)

Table 3. Specifications of samples used for phylogenetic analyses.

No.	Year	Season	State	Age (years)	Sex	ID in the Phylogenetic tree
3	2018	Dry	PB	3	Male	-
7	2018	Dry	PB	4	Male	7_Eq_Cajazeiras_PB_BR_2018
39	2017	Dry	CE	3	Female	39_Eq_Aquiraz_CE_BR_2017
42	2017	Dry	CE	6	Female	42_Eq_Jaguaribara_CE_BR_2017
45	2017	Dry	CE	6	Female	-
51	2017	Dry	RN	8	Male	51_Eq_Mossoro_RN_BR_2017
72	2017	Dry	CE	1	Female	72_Eq_Mauriti_CE_BR_2017
104	2017	Rainy	RN	1	Female	104_Eq_Caico_RN_BR_2017
115	2018	Rainy	PE	2	Female	-
129	2017	Rainy	PB	8	Male	129_Eq_Imaculada_PB_BR_2017
134	2017	Dry	PB	6	Male	134_Eq_Santa_Rita_PB_2017
150	2017	Rainy	PB	9	Male	-
155	2017	Rainy	PB	2	Male	155_Eq_Patos_PB_BR_2017
168	2017	Rainy	CE	3	Female	168_Eq_Fortaliza_CE_BR_2017
169	2018	Rainy	PB	4	Female	169_Eq_Cajazeiras_PB_BR_2018
171	2018	Rainy	PB	11	Female	-
172	2018	Rainy	PB	15	Male	-

F: female, M: male, PB: Paraíba, CE: Ceará, RN: Rio Grande do Norte, PE: Pernambuco; - Unsequenced

Fig.1

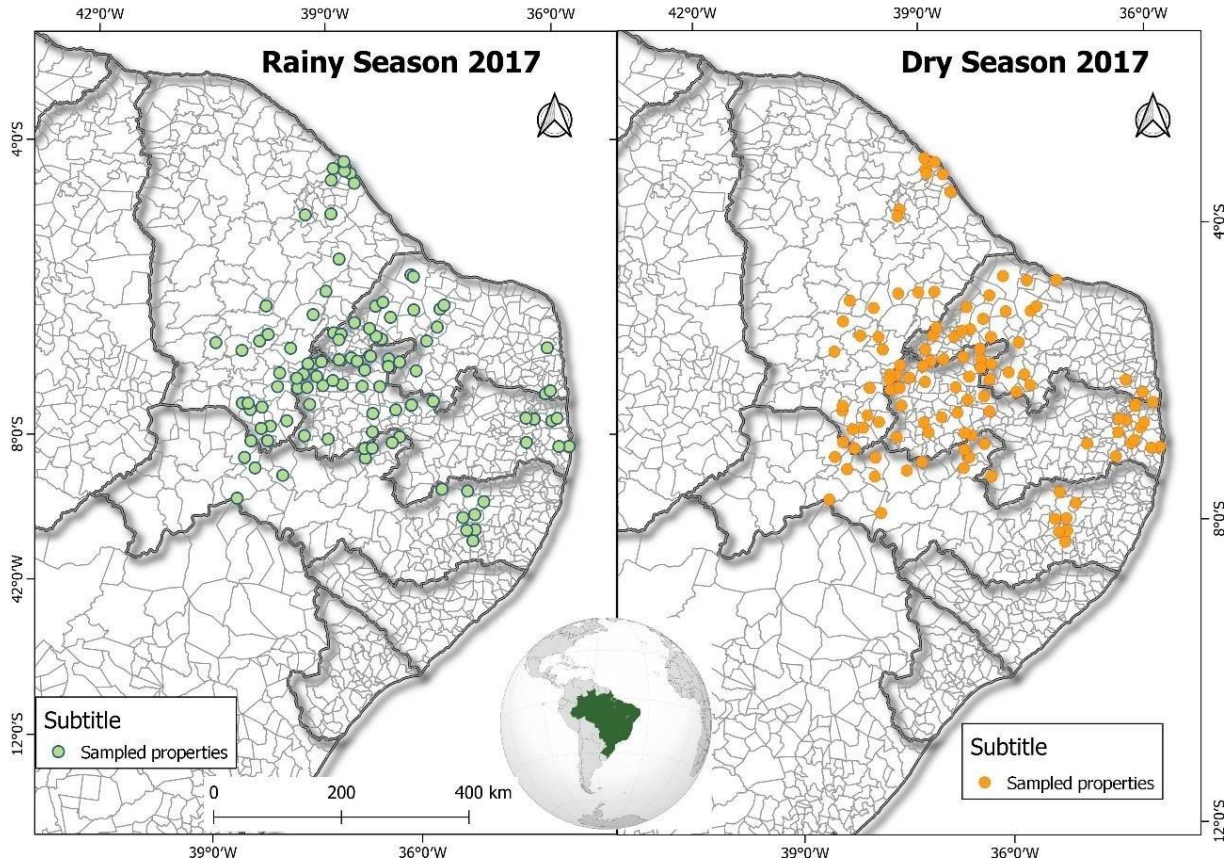
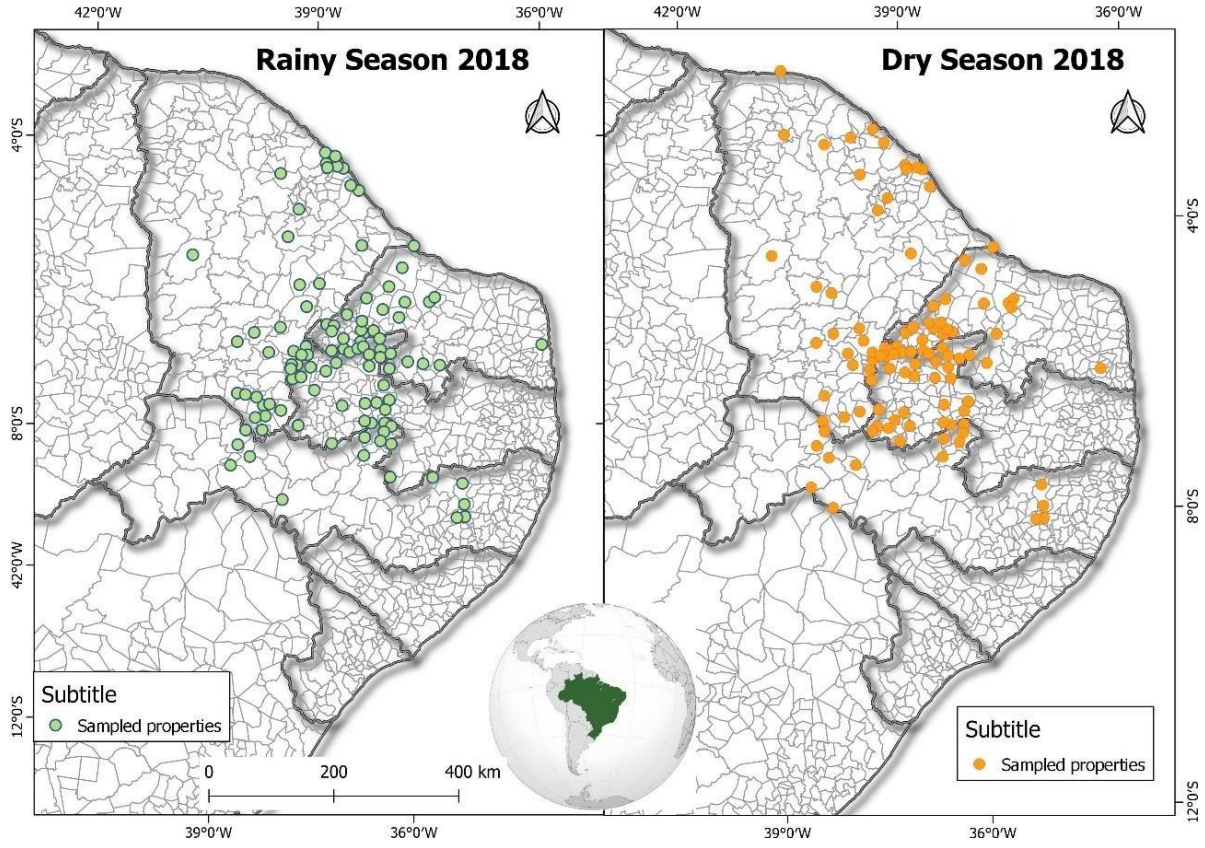

Rainy Season 2017
Dry Season 2017
42°0'W
39°0'W
36°0'W
4°0'S
8°0'S
12°0'S
Subtitle
Sampled properties
0
200
400 km

Fig.2


Rainy Season 2018
Dry Season 2018
42°0'W
39°0'W
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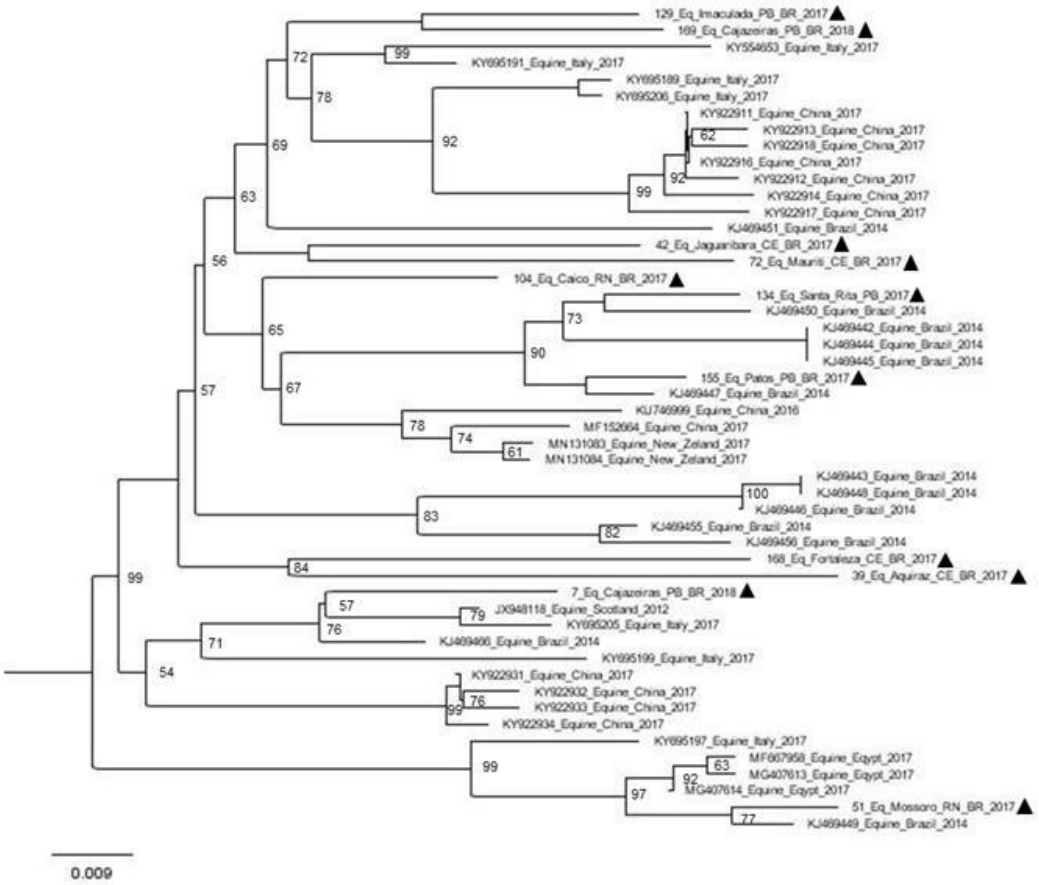
0
200
400 km

Fig.3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KY554653_Equine_Italy_2017
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KY922916_Equine_China_2017
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KY922914_Equine_China_2017
KY922917_Equine_China_2017
KJ469451_Equine_Brazil_2014
42_Eq_Jaguaribara_CE_BR_2017
72_Eq_Mauriti_CE_BR_2017
104_Eq_Caico_RN_BR_2017
134_Eq_Santa_Rita_PB_2017
KJ469450_Equine_Brazil_2014
KJ469442_Equine_Brazil_2014
KJ469444_Equine_Brazil_2014
KJ469445_Equine_Brazil_2014
155_Eq_Patos_PB_BR_2017
KJ469447_Equine_Brazil_2014
KU746999_Equine_China_2016
MF152664_Equine_China_2017
MN131083_Equine_New_Zeland_2017
MN131084_Equine_New_Zeland_2017
KJ469443_Equine_Brazil_2014
KJ469448_Equine_Brazil_2014
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KJ469455_Equine_Brazil_2014
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168_Eq_Fortaleza_CE_BR_2017
39_Eq_Aquiraz_CE_BR_2017
7_Eq_Cajazeiras_PB_BR_2018
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CAPÍTULO III:

Título do capítulo III

Global prevalence of equine hepacivirus (*EqHV*): systematic review and meta-analysis

(Journal of Equine Veterinary Science, Qualis: B2, Fator de Impacto: 1.583)

REVIEW ARTICLES

Global prevalence of equine hepacivirus (*EqHV*): systematic review and meta-analysis

Camila de Sousa Bezerra[a], Clécio Henrique Limeira[a], Denize Monteiro dos Anjos[b], Denise Batista Nogueira[c], Davidianne de Andrade Morais[a], Clebert José Alves[a], Carolina de Sousa Américo Batista Santos[a,] Maria Luana Cristiny Rodrigues Silva[a], Sérgio Santos de Azevedo[a*].

[a]Federal University of Campina Grande (UFCG), Patos, Paraíba, Brazil
[b]Federal University of Paraíba (UFPB), João Pessoa, Paraíba, Brazil
[c]Federal University of São Paulo (USP), São Paulo, São Paulo, Brazil
[*]CORRESPONDENCE: S.S. de Azevedo [sergio.santos@professor.ufcg.edu.br]. Department of Preventive Veterinary Medicine - UFCG. Avenida Universitária s/n, Santa Cecilia, CEP 58708-110, Patos, PB, Brazil.

Abstract

Equine hepacivirus (*EqHV*) belongs to the *Flaviviridae* family, genus *Hepacivirus* and has the greatest genomic identity with the hepatitis C virus (HCV), one of the main causes of chronic liver disease in humans. Due to the limited applicability of studies of *HCV* in animal hosts, the interest in studies of characterization of viral homologues has been growing. For this reason, we performed a systematic review of the literature with meta-analysis of the prevalence of *EqHV* and genetic sequencing studies. Twenty-three studies from five different continents were analyzed. The combined prevalence of positive animals was 7.01% (95% CI 4.64 - 10.47%), with the highest proportions in Asia (14.27%; 95% CI 6.34 - 29.03%), followed by South America (12.03%; 95% CI 9.58 - 15.01%), Africa (8.69%; 95% CI 6.71 - 11.20%), Europe (3.63%; 95% CI 2.10 - 6.22%) and North America (2.87%; 95% CI 1.91 - 4.28%). Variables associated with *EqHV* infection were indirectly related to animal management such as transport, reproductive practices, among others. Therefore, preventive measures must aim at sanitary control of the sources of viral infection.

Keywords: Equine Hepacivirus; Non-primate Hepacivirus; Prevalence; Meta-analysis.

1. Introduction

Hepatitis C virus (*HCV*) belongs to the *Flaviviridae* family, genus *Hepacivirus*, with humans as natural hosts and chimpanzees as experimental hosts [1]. *HCV* is the etiologic agent of hepatitis C in humans, one of the main causes of chronic liver disease worldwide [2]. Several homologs of *HCV* have been identified in animal hosts, such as canines [3], horses [4], mules [5], cattle [6], bats [7], rodents [8], non-human primates [9] and sharks [10]. Among the 14 viral species of the *Hepacivirus* genus [11], equine hepacivirus (*EqHV*), also called *HAV*, has the highest genomic identity with *HCV* [12], suggesting the presence of a common ancestor not yet identified [13].

Among the similarities between *EqHV* and *HCV* is the presence of an open reading frame (ORF) in the viral genome, which encodes approximately 2940–2950 amino acid polyproteins, which produce non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) and structural (core, E1, and E2) [14]. Furthermore, similar towhat occurs in *HCV* infection, alterations in liver functions have already been observed [15, 16, 17, 18] and the development of a chronic picture in animals infected with EqHV [19, 20, 21].

The first report of *EqHV* occurred in the USA in 2012, from dogs with respiratory diseases, being initially called canine hepacivirus (*CHV*) [3]. However, horses were later shown to be the primary hosts of *EqHV* [12]. Since then, its circulationhas been described in different countries, such as the United States [18], Brazil [22, 23],South Africa [4], Morocco [24], Japan [25], China [26], South Korea [27], Mongolia [28], Italy [21], France [20], Germany [8, 29], United Kingdom [15] and Hungary [30].

Studies aimed at the development of vaccines and antiviral therapies for *HCV* have limited applicability, due to the unavailability of animal models, since, for ethical reasons, the use of chimpanzees is prohibited in many countries. For this reason, characterization studies of viral homologs are useful for understanding the pathogenesis of *HCV* and for choosing an animal model to study the infection. Given the importance of characterizing hepacivirus infections in domestic animals for the understanding of hepatitis C in humans, and the genetic proximity between *HCV* and *EqHV*, a systematic literature review was carried out with a meta-analysis of prevalence and sequencing studies genetic of the *EqHV*.

2. Methodology

2.1. Study design

A systematic literature review was carried out with emphasis on the prevalence of equine hepacivirus (*EqHV*), followed by a meta-analysis of quantitative data. For the preparation of this study, the PRISMA methodology recommendations were observed - Preferred Reporting Items for Systematic Reviews and Meta-analyses [31].

2.2. Search strategies

The search for primary studies was performed in the following electronic databases: MEDLINE/PubMed, Science Direct, Scopus, and Web of Science, using the terms in English: {Equine Hepacivirus} OR {Hepacivirus A} OR {Non-primate hepacivirus} AND {prevalence}. The citations of the identified studies containing the title and abstract were saved in BibTex format and viewed in the Mendeley® bibliographic manager, in which duplicated works were excluded and the titles and abstracts were read.

2.3 Article eligibility

In the selection of studies, there was no restriction regarding the year of completion or publication, the language of publication of the article, or the country of development of the research. Full articles, case reports, brief communications, sequence records, and letters were selected. Book chapters and appendices were excluded from the selection. The surveys were carried out between the 9th and 15th of November 2021.

2.4. Selection of studies and data extraction

The selection of studies was performed by two researchers independently, initially analyzing the title and abstract and, subsequently, reading the articles individually. Disagreements about its inclusion were resolved by consensus between the two researchers. The following inclusion criteria were pre-established: (1) *EqH*V prevalence studies; (2) PCR as a direct diagnosis. Studies describing other viral agents without determining the interference of each of them in the prevalence values and studies focusing on aspects of viral molecular structures were excluded, as well as studies that used only serological tests for diagnosis. Data extraction and analysis

The data extracted from the articles were added to an Excel spreadsheet, containing the following information: author, year of publication, country, continent,

number of animals tested, number of positive animals, prevalence, diagnostic method, sequenced genome region, biochemical alterations, risk factor and/or association.

2.5 Data analysis

A meta-analysis was performed with all primary studies included, considering the prevalence of *EqHV* infection as an outcome. Another meta-analysis was performed using the variable sex (male or female) as a factor associated with *EqHV* infection, using data from primary studies to calculate the Odds Ratio (OR). Other risk factors identified in the studies were analyzed descriptively.

Heterogeneity was determined by the Cochran Q test and quantified by the Higgins and Thompson I^2 test. The combined prevalence estimates and 95% CI were calculated based on the random effects model by the inverse of variance, using the DerSimonian-Laird method. The presence of publication bias was analyzed by viewing the funnel graph and applying the Egger test. All analyzes were conducted using the statistical program R version 3.5.1 [32].

3. Results

The initial search process in the four electronic databases returned a total of 226 results, of which 23 met the predefined criteria and were included in this review. The search and selection steps are outlined in Figure 1.

The 23 studies were carried out in countries on five different continents: Europe (n = 10), Asia (n = 7), South America (n=3), Africa (n=2), and North America (n=1), and published between 2012 and 2021. The main information and data from surveys on the prevalence of *EqHV* are available in Table 1.

The initial meta-analysis showed a combined prevalence of *EqHV* infection of 7.01% (95%CI 4.64 - 10.47%), but with the presence of heterogeneity identified by the Cochran Q test ($p<0.01$) and classified as high heterogeneity by the Higgins and Thompson test ($I^2 = 97.0\%$). Thus, it was decided to carry out a meta-analysis by subgroup, according to the continent where the research was carried out, using the random effects model, which incorporates the heterogeneity of the studies in the combination of prevalence, to try to identify the source of the heterogeneity (Figure 2).

When analyzing the results of the meta-analysis by continents (Table 2 and Figure 2), it was observed that the prevalence of *EqHV* infection was higher in Asia (14.27%; 95% CI = 6.34 - 29.03%), followed by from South America (12.03%; 95% CI = 9.58 - 15.01%), Africa (8.69%; 95% CI = 6.71 - 11.20%), Europe (3.63%; 95% CI = 2.10 - 6.22%) and North America (2.87%; 95% CI = 1.91 - 4.28%).

In addition to prevalence, risk factors and variables associated with *EqHV* infection in horses were analyzed. Figure 3 shows a forest graph that shows the combination of the odds ratio (OR) of seven studies with respect to the sex of the animals. Other factors associated with infection were summarized and can be seen in Table 3. Biochemical changes observed in animals infected with *EqHV* in some studies are summarized in Table 4.

The funnel graph visualization (Figure 4) points to an asymmetric distribution of the 23 studies, suggesting the presence of publication bias. However, when applying the Egger test (P = 0.09), the occurrence of such biases was not verified.

4. Discussion

Twenty-three articles were selected for systematic review. Due to the recent identification of *EqHV*, the number of studies that met the pre-established selection criteria was low. The first report of *EqHV* occurred in 2012 in the USA, from dogs with respiratory disease, initially called canine hepacivirus (*CNV*) [3]. It was later identified that horses are the primary hosts of the viral species [12].

Regarding the region of choice for molecular diagnosis and genetic sequencingof *EqHV*, most studies used the nucleotide sequences of the NS5B and/or NS3 genes.As they are highly conserved, phylogenetic analysis based on these regions produces results similar to those obtained from complete genome sequencing [1].

To identify the reason for the high heterogeneity found in the meta-analysis, the subdivision of works was carried out according to the continent where the research was carried out. There was a significant reduction in the heterogeneity of results in the African (p = 0.31; I2 = 1%) and South American (p = 0.29; I2 = 19%) continents. The highest continental prevalences of *EqHV* positive animals were observed in Asia (14.27%; 95%CI 6.34 - 29.03%), South America (12.03%; 95%CI 9.58 - 15.01%), and Africa (8.69%; 95%CI 6.71 - 11.20%). Such results may be related to the management

of animals in these regions, since the main forms of *EqHV* transmission known to date are iatrogenic and vertical [33, 34].

Considering the variable gender of the animals with the occurrence of positive cases, there was no significant difference in the OR values between the analyzed studies (Figure 3), however, such association was described in some individual studies, as shown in Table 3. In three articles, females showed a higher prevalence of *EqHV*- positive animals than males, supposedly due to reproductive management practices in these animals, thus favoring iatrogenic and/or vertical transmission [17, 24, 35]. In just one study analyzed, males had a higher proportion of positivity, however, it was not possible to correlate this variable to a plausible cause of the prevalence obtained [4].

Still on the variables associated with *EqHV* infection, in the studies included in this review, a higher prevalence of positive cases was observed among young animals, except in the work by Wu et al. [35] in which animals ≥10 years old had a greater proportion of positive horses. Badenhorst et al. [4] found that horses >5 months were more likely to be positive for *EqHV*, correlating with a reduction in maternal anti-*EqHV* antibodies, as these decrease between three and six months of age [33]. In two studies, the highest prevalence of positive horses in young animals was linked to frequent handling practices in animals of this age group, whether for reproductive purposes (<4 years) [17] or for participation in races (1 – 2 years) [25]. Reichert et al. [36] found that younger horses (<8 years) had a higher risk of *EqHV* infection, combined with a history of international transport, due to stress-induced immunosuppression during handling of these animals.

Thoroughbred has been identified as a variable associated with the highest prevalence of *EqHV* positive animals in several studies and may be related to the handling, displacement or genetic susceptibility [20]. Thoroughbred horses are mainly used in equestrian racing and for this reason, they are often transported to other regions, which could favor the introduction of *EqHV* into susceptible herds. Another important fact is that artificial insemination is not allowed in the reproduction of thoroughbred requiring the displacement of these animals during the breeding season [16, 27]. Repeated management practices in thoroughbred could favor iatrogenic transmission by equine-derived material, as already *EqHV* has been isolated in commercial serum [20, 29].

Finally, in one study, warmblood horses had higher proportions of positive *EqHV* cases than the others [35]. So-called “warm-blooded” horses comprise horses of a

lively and fast breed, such as thoroughbred for example. This term can also be used for the result of mating a thoroughbred or Arabian animal. Thus, the hypotheses for the identification of PSI animals as a variable associated with *EqHV* infection could be indirectly applied to the so-called warmblood animals.

During the individual reading of the articles, the hepatic biochemical alterations described in the selected studies were searched (Table 4). *EqHV* positive animals showed slight increase of glutamate-oxaloacetate transaminase (GOT), glutamic pyruvic transaminase (GPT) [23], aspartate aminotransferase (AST) [17], sorbitol dehydrogenase (SDH) [18], glutamate dehydrogenase (GLHD) and gamma- glutamyltransferase (GGT) [15, 16, 17, 18, 19, 25]. Mild increase of GGT levels were identified in more than one article, except one *EqHV* positive animal that had a highly increased GGT level [16].

Changes in liver parameters have been described in experimentally infected animals, containing high *EqHV* viral loads [19, 37]. However, high levels of biochemical parameters in naturally infected horses are uncommon [25] or occur discreetly. Thus, it is likely that animals infected with *EqHV* are asymptomatic, with no significant changes in enzyme values [18].

In the funnel plot (Figure 4), each point represents a study, with the prevalence organized on the X-axis and the standard error on the Y-axis. In the absence of publication bias, the points are expected to have a symmetrical distribution under the area of the triangle. Thus, the visual assessment of the figure demonstrates an asymmetric distribution of points (studies), indicating a possible publication bias in this meta-analysis. However, when applying the Egger test ($P = 0.09$), the occurrence of such biases was not verified, and the asymmetry of the funnel graph can be attributed to other factors, such as the high heterogeneity found [38].

5. Conclusion

This systematic literature review addressed the main information on *EqHV* infection in horses from various parts of the world. Due to the ethical limitations of *HCV* research in susceptible animals, the interest in analyzing aspects of viruses homologous to *HCV* has been growing, and for this reason, the data presented in this review can support the understanding of the infection in humans.

The variability of prevalence obtained by continent may be correlated with the applicability of sanitary measures to prevent the transmission of infectious agents, since more developed areas had lower proportions of positive animals, while continents with a higher prevalence of *EqHV* positive animals are considered emerging. The variables associated with *EqHV* infection are indirectly related to animal management such as transport, reproductive practices, among others. For this reason, preventive measures must be aimed at sanitary control of the sources of viral infection.

Acknowledgments

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Figure 1 – Flowchart of the search and selection process of primary studies included in the systematic review.

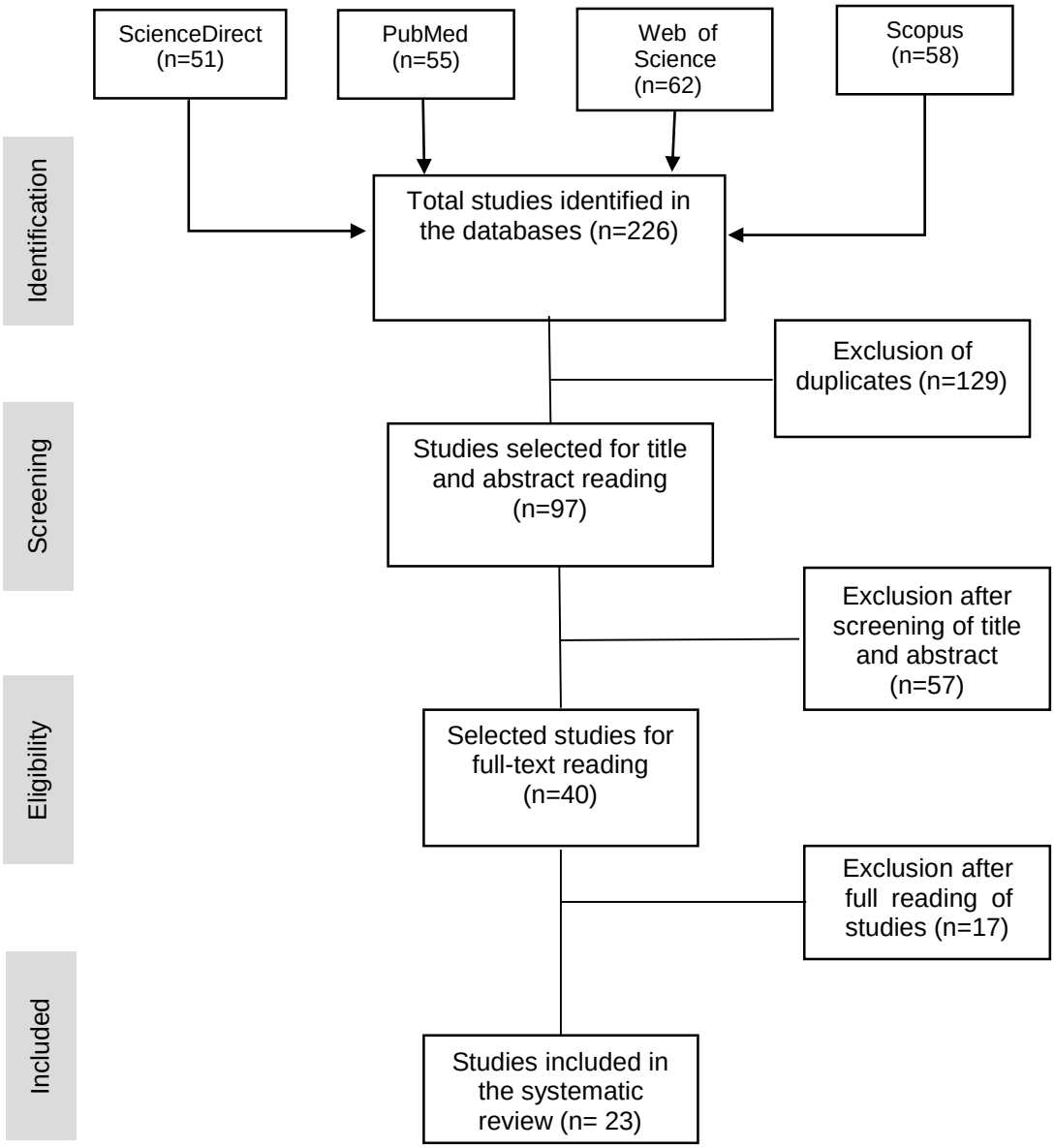

Figure 2 - Forest plot of the meta-analysis of the prevalence of *EqHV* infection in horses by subgroup, according to the continent where the survey was carried out.

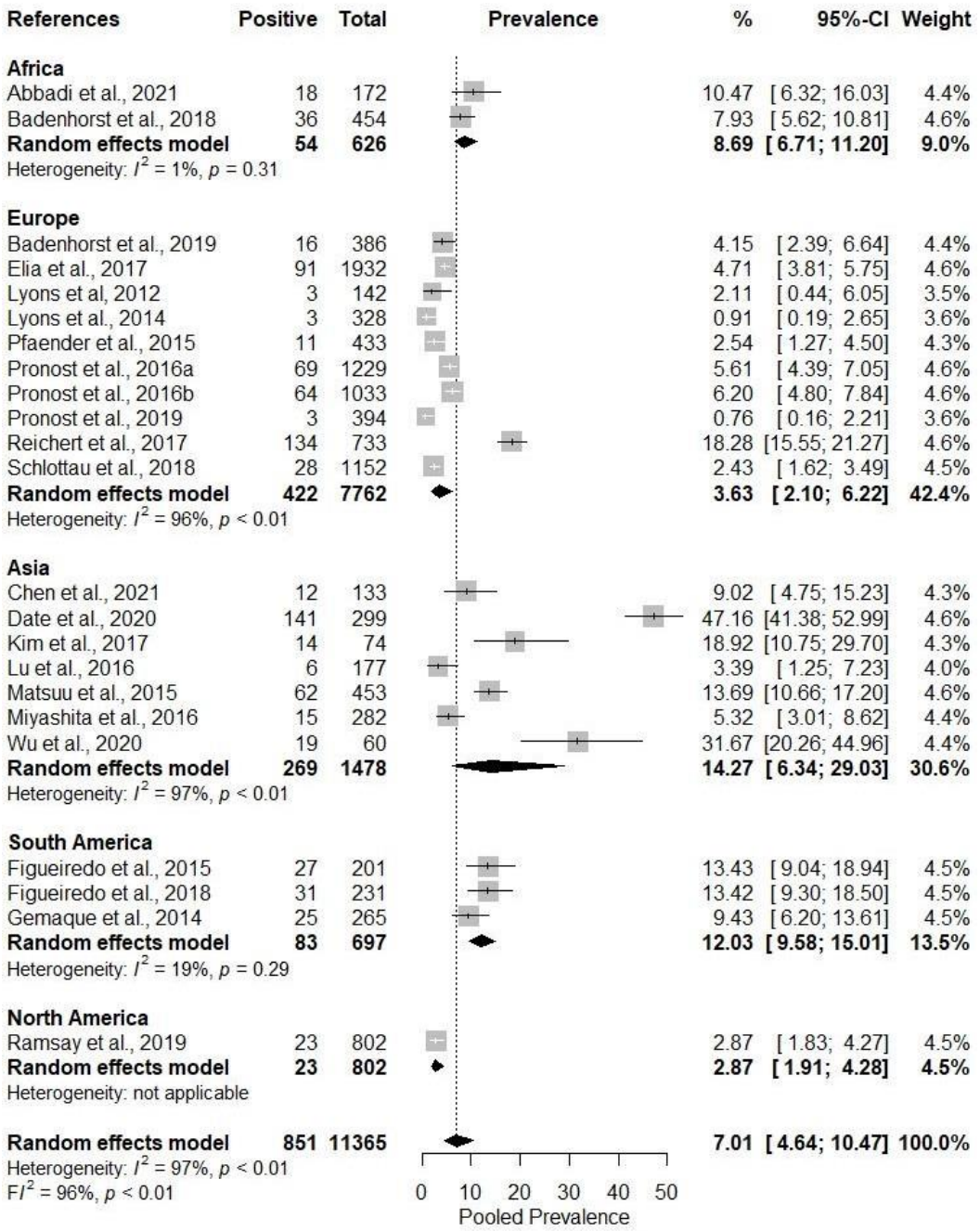

Figure 3 – Forest plot of the meta-analysis demonstrating the OR according to the sex of the animals

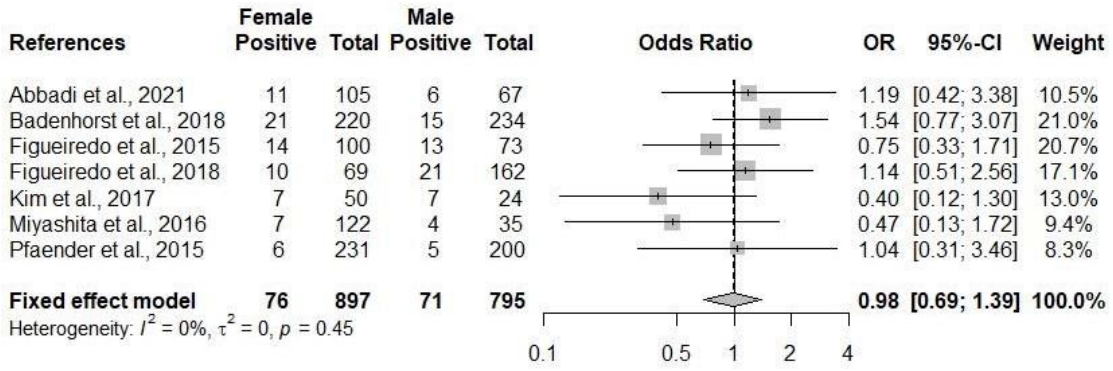

Figure 4 - Funnel plot demonstrating the distribution of studies (points) according to Logit prevalence and standard error, as a way to visualize the presence of publication bias.

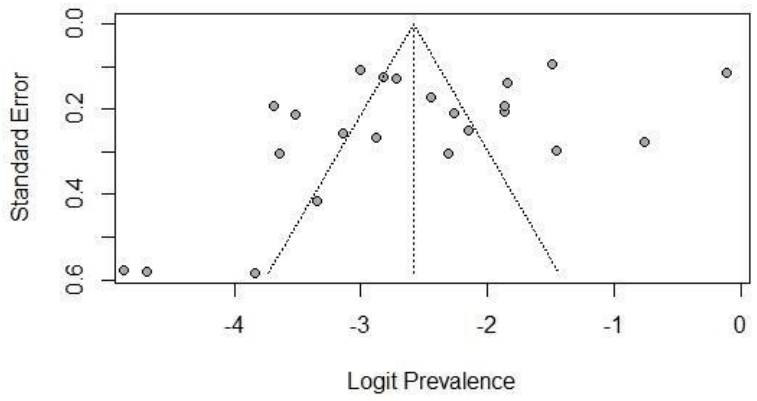

Table 1 - Summary of key information from the 23 studies included in the systematic review of the prevalence of *EqHV* in horses

References		Location		Results				Region of genetic sequencing
Author	Year	Country	Continent	Sample	Positives	Prevalence (%)	CI 95%	
Abbadi et al.,	2021	Morocco	Africa	172	18	10.5	6.3 - 16.0	NS3
Badenhorst et al.,	2018	South Africa	Africa	454	36	7.9	5.6 - 10.8	5′UTR, NS3 and NS5B
Badenhorst et al.,	2019	Austria	Europe	386	16	4.2	2.4 - 6.6	5'UTR, NS3 and NS5B
Chen et al.,	2021	China	Asia	133	12	9.0	4.7 - 15.2	NS3
Date et al.,	2020	Mongolia	Asia	299	141	47.2	41.4 - 52.9	5'-UTR, core, NS3, NS5B, and full genome
Elia et al.,	2017	Italy	Europe	1932	91	4.7	3.8 - 5.8	NS3, NS5B and 5′ UTR
Figueiredo et al.,	2015	Brazil	South America	201	27	13.4	9.3 - 18.5	NS5B
Figueiredo et al.,	2018	Brazil	South America	231	31	13.4	9.3 - 18.5	NS5B
Gemaque et al.,	2014	Brazil	South America	265	25	9.4	6.2 - 13.6	NS3
Kim et al.,	2017	Korea	Asia	74	14	18.9	10.7 - 29.7	NS3
Lu et al.,	2016	China	Asia	177	6	3.4	1.3 - 7.2	5'UTR, NS3 and NS5B
Lyons et al,	2012	Scotland	Europe	142	3	2.1	0.4 - 6.0	-
Lyons et al.,	2014	Scotland, England and France	Europe	328	3	0.9	0.2 - 2.6	-
Matsuu et al.,	2015	Japan	Asia	453	62	13.7	10.7 - 17.2	NS3
Miyashita et al.,	2016	Japan	Asia	282	15	5.3	3.0 - 8.6	NS3
Pfaender et al.,	2015	Germany	Europe	433	11	2.5	1.3 - 4.5	NS3
Pronost et al.,	2016	France	Europe	1229	69	5.6	4.4 - 7.1	5'UTR, NS3 and NS5B
Pronost et al.,	2016	France	Europe	1033	64	6.2	4.8 - 7.8	5'UTR, NS3 and NS5B
Pronost et al.,	2019	France	Europe	394	3	0.76	0.2 - 2.2	5'UTR, NS3 and NS5B
Ramsay et al.,	2019	USA	North America	802	23	2.9	1.8 - 4.3	-

Reichert et al.,	2017	Germany	Europe	733	134	18.2	15.5- 21.3	-
Schlottau et al.,	2018	Germany	Europe	1152	28	2.4	1.62 - 3.5	NS3
Wu et al.,	2020	China	Asia	60	19	31.7	20.3 - 44.9	5′-UTR, ORF and 3'UTR

Table 2 - Summary of the meta-analysis of the prevalence of *EqHV* infection in horses, by subgroup according to the continent where the survey was carried out.

	Number of studies	Sample	Positives	Pooled prevalence (95%-CI)	Heterogeneity	
					p-value	I^2
Pooled overall prevalence	23	11365	851	7,01% (4,64 - 10,47%)	< 0.01	97%
Continent						
Africa	2	626	54	8,69% (6,71 -11,20%)	0.31	1,1%
Europe	10	7762	422	3,63% (2,10% - 6,22%)	<0.01	96,1%
Asia	7	1478	269	14,27% (6,34 - 29,03%)	<0.01	96,9%
South America	3	697	83	12,03% (9,58 - 15,01%)	0.29	19,1%
North America	1	802	23	2,87% (1,91 - 4,28%)	Not applicable	

Table 3 – Risk factors or associated variables to *EqHV* infection in horses cited in primary studies included

Risk factor/association	References
Female	Abbadi et al., 2021
Colts	Badenhorst et al., 2018
> 5 months	
< 4 years	Figueiredo et al., 2018
Female	
Thoroughbred	Kim et al., 2017
1–2 years	Matsuu et al., 2015
Thoroughbred	Pfaender et al., 2015
Thoroughbred	Pronost et al., 2016
< 8 years + international transport	Reichert et al., 2017
Female	
≥ 10 years	Wu et al., 2020
Warmblood	

Table 4 – Biochemical changes observed in horses infected with *EqHV*

References	Biochemical changes
Badenhorst et al., 2018	Increased GLDH
Figueiredo et al., 2015	Increased GOT and GPT
Figueiredo et al., 2018	Increased AST and GGT
Lyons et al., 2012	Increased GGT
Matsuu et al., 2015	Increased GGT
Pfaender et al., 2015	Increased GGT
Ramsay et al., 2019	Increased SDH and GGT

AST = Aspartate transaminase; GGT = Gamma-glutamyl transferase; GLDH = Glutamate dehydrogenase; GOT = Glutamic-oxaloacetic transaminase; GPT = Glutamate pyruvate transaminase; SDH = Succinate dehydrogenase.

CONCLUSÃO GERAL

Com base no que foi apresentado, os três capítulos que compõe esta Tese levaram às seguintes conclusões:

- O *EIAV* é circulante em equinos dos estados da Paraíba, Rio Grande do Norte, Ceará e Pernambuco, região Nordeste do Brasil;
- O Ceará apresentou a maior prevalência de animais *EIAV* positivos, bem como áreas com maior densidade de animais positivos;
- As regiões de fronteira entre os estados foram as áreas com os maiores conglomerados de casos positivos para o *EIAV*, sendo necessário reforçar o controle da movimentação dos animais e as medidas preventivas de infecção nesses locais;
- A circulação do *EqHV* foi evidenciada na região Nordeste do Brasil;
- A identidade próxima das cepas isoladas nos estados da PB, RN, PE e CE com outras sequências brasileiras, sugerem a presença de um ancestral comum;
- A variabilidade das prevalências do *EqHV* obtidas por continente pode estar correlacionada com a aplicabilidade de medidas sanitárias nessas áreas;
- As variáveis associadas à infecção pelo *EqHV* estão indiretamente relacionadas ao manejo dos animais como transporte, práticas reprodutivas, entre outros.